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14. ABSTRACT

My experiments indicate that Serum and Glucocorticoid regulated Kinase (SGK) proteins facilitate breast cancer invasive migration, a critical step for ultimate cancer metastasis to other organs. The activation of SGK during cellular stress conditions, such as low oxygen found within a tumor, makes this data increasingly imperative for therapeutics. The research shown here demonstrates SGK loss in highly mestatic breast cancer cell lines causes an invasive migration defect. Conversely, the overexpression of SGK isoforms in breast cancer cell lines causes an enhancement of invasive migration. These discoveries are helping to elucidate new mechanisms that can be targeted for more specific and successful therapies to block breast cancer metastasis. Known targets of SGK proteins are being examined for their contribution to the metastatic properties of breast cancer cells. These studies will determine the importance of SGK proteins as putative therapeutic targets for breast cancer motility.

15. SUBJECT TERMS
Serum and Glucocorticoid Regulated Kinase (SGK), breast cancer cell migration and invasion, retroviral overexpression, lentiviral silencing

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Introduction

Breast cancer arises as a result of alterations in the cell signaling networks that control growth, division, and metastasis. Knowledge of the details of these signaling systems is becoming increasingly essential for the design of new and effective treatments. One such pathway is phosphoinositide 3-kinase (PI3K) which activates numerous downstream effectors, which in turn regulate a plethora of physiological and pathophysiological process. In human cancer, two critical PI3K effectors are the protein kinases Akt, the human homologue of the viral oncogene *v-Akt,* and SGK, the Serum and Glucocorticoid-regulated Kinase. The importance of this pathway in disease is underscored by the fact that the gene encoding the catalytic subunit of PI3K, *PIK3CA*, is one of the most frequently mutated oncogenes in breast cancer. The two most common *PIK3CA* somatic oncogenic mutations occurring in breast cancer are E542K, in the helical domain, and H1047R, in the kinase domain, and both mutations result in hyperactive PI3K signaling (1). The oncogenic *PIK3CA* mutations have been documented at a frequency 25-40% in breast tumors (2,3). Therefore, evaluating the function of downstream effectors of PI3K in breast cancer, such as Akt, has been a cancer biology focus for some time. Numerous studies have revealed PI3K and Akt as a central signaling node integrating processes of cell growth, survival, and proliferation. Recently our laboratory discovered that distinct isoforms of Akt (Akt1 and 2) also play a key role in breast cancer cell invasive migration, whereby Akt1 inhibits and Akt2 enhances invasive migration (4). Despite the wealth of knowledge concerning PI3K and Akt signaling, relatively little is known about SGK, also a PI3K effector. SGK isoforms share many characteristics with Akt, such as high homology in the catalytic domain, common mechanism of phosphorylation and activation, a significant number of overlapping substrates, and deregulation in breast carcinoma (5,6). To date, no studies have investigated any role for SGK in cell invasive migration or metastasis. This is despite the fact that SGK is also activated by oncogenic PI3K and that SGK isoforms are documented as amplified with high frequency in breast cancer(6). This makes the similarities and distinctions between Akt and SGK attractive targets for therapeutic applications in cancer.

Body

The following tasks from the Statement of Work for this project were the focus for the research period from March 15, 2011- March 14, 2012: Examine the influence of SGK isoforms on cellular motility both individually and in concert with Akt isoforms (months 1-27)

1.4 Overexpress SGK and Akt isoforms individually in normally migratory breast cancer cell lines and normally static breast cell lines and evaluate motility changes via Transwell migration and Transwell Matrigel invasion assays (months 8-10)

1.5 Overexpress SGK isoforms in concert with Akt isoforms and evaluate motility changes with Transwell migration and Transwell Matrigel invasion assays in normally migratory breast cancer cell lines and normally static breast cell lines (months 11-13)

1.6 Silence SGK expression, individually and in concert with Akt, using lentiviral siRNA constructs and evaluate motility changes with Transwell migration and Transwell Matrigel invasion assays in normally migratory breast cancer cell lines and normally static breast cell lines (months 14-17)

1.7 Run Transwell migration assays and Transwell Matrigel invasion assays using PIK3CA mutant MCF10A cells and optimized SGK1/3 and Akt1 and Akti siRNA (months 18-21)

1.8 Make SGK known substrate phosphorylation mutants and optimize their expression (month 22-23)

1.9 Run Transwell migration assays and Transwell Matrigel invasion assays with SGK substrates mutants in background of SGK overexpression and silencing to examine SGK substrate invasive migration importance to SGK invasive migration phenotype (months 24- 27)

Progress

1.2 Optimize lentiviral infection technique and siRNA silencing sequences for Akt and SGK isoforms in breast carcinoma cell lines (months 2-3)

Silencing of SGK and Akt isoforms had been confirmed March 15, 2010- March 14, 2011. However on further analysis of substrate NDRG1 it appears the silencing of SGK3 but not Akt isoforms causes an increase in total NDRG1 protein level while simultaneously decreasing phosphorylation of NDRG1 at T346/356/366 SGK phosphorylation sites (further discussed in aim 1.8) (FIGURE 1).

1.3 Optimize lentiviral infection for PIK3CA and refractory PIK3CA overexpression in MCF10A breast cells (month 4-7)

March 15, 2010- March 14, 2011 I had identified 2 PIK3CA/pLKO shRNA constructs that efficiently silenced PIK3CA expression and transfection overexpression system that worked efficiently in 293T cells. At the start of March 15, 2011 we tested the same overexpression vectors of PIK3CA and its two most common breast cancer mutation hotspots, E545K and H1047R in MCF10A breast cells but expression was insufficient to get measurable signal of SGK3 activity by western blot. We then regressed to use 293T cells for overexpression and in vitro kinase assays to confirm the effect of p110a activity upon SGK3 kinase activity. (FIGURE 2). Upon transfection of E545K and H1047R PIK3CA mutations in 293T cells a significant increase the activity of exogenous SGK3 was observed measured by SGK3 pulldown and in vitro kinase assay upon known substrate GST-GSK3beta and its phosphorylation at Serine 9 known SGK3 site.

1.6 Silence SGK expression, individually and in concert with Akt, using lentiviral siRNA constructs and evaluate motility changes with Transwell migration and Transwell Matrigel invasion assays in normally migratory breast cancer cell lines and normally static breast cell lines (months 14-17). SGK3 shRNA and Akt 2 shRNA both dramatically inhibit MCF7 breast cancer cell migration although SGK3 shRNA inhibits migration to a greater extent than Akt2 shRNA (FIGURE 3). The difference in SGK3 and Akt2shRNA inhibition is statistically significant. Upon dual knockdown of Akt2 and SGK3 upon co-infection with shRNAs and selection the inhibition is equivalent to SGK3 knockdown alone and does not inhibit to a greater extent. This could be explained by redundant substrates or that the level of migration was already inhibited to its maximum by SGK3 alone. To further examine the redundancy of the two kinases both SGK3 and Akt2 will be silenced in a cell line that exhibits higher degrees of migration such as T47D breast cancer cells, which will thus enable us to identify a greater number of differences in invasive migration inhibition and substrate regulation.

1.8 Make SGK known substrate phosphorylation mutants and optimize their expression (month 22-23) NDRG1 has been shown to be a potent mediator of invasive migration through its roll in the recycling of E-cadherin the cellular membrane(7). However, the roll of SGK3 phosphorylation in the regulation of NDRG1 activity was not known. We mutated the 5 SGK phosphorylation sites in NDRG1-FLAG to T328A/T330A/T346A/T356A/T366A and were able to see reproducible overexpression with transient transfection 293T cells (FIGURE 4). Upon SGK3 silencing we were able to see an increase in total NDRG1 with a simultaneous decrease in phosphorylation (FIGURE 1). We therefore examined the stability of NDRG1 with cyclohexamide treatment and were able to see a decrease in NDRG1 levels with the inhibition of protein synthesis in MCF10A breast cancer

cells (FIGURE 5). We then treated MCF10A cells with MG132 and were able to see a significant increase in total NDRG1 with inhibition of 26S proteasome degradation activity (FIGURE 6).

1.9 Run Transwell migration assays and Transwell Matrigel invasion assays with SGK substrates mutants in background of SGK overexpression and silencing to examine SGK substrate invasive migration importance to SGK invasive migration phenotype (months 24- 27)

NDRG1 wildtype was initially run in a migration assay in MCF7 breast cancer cells in order to confirm previously data implicating NDRG1 as an inhibitor of migration. NDRG1 significantly inhibited MCF7 migration. With the addition of SGK inhibitor GSK650394 MCF7 cells were inhibited to a greater extent thus implying SGK3 may be enhancing migration through its enhancement of NDRG1 degradation (FIGURE 7).

FIGURE 1

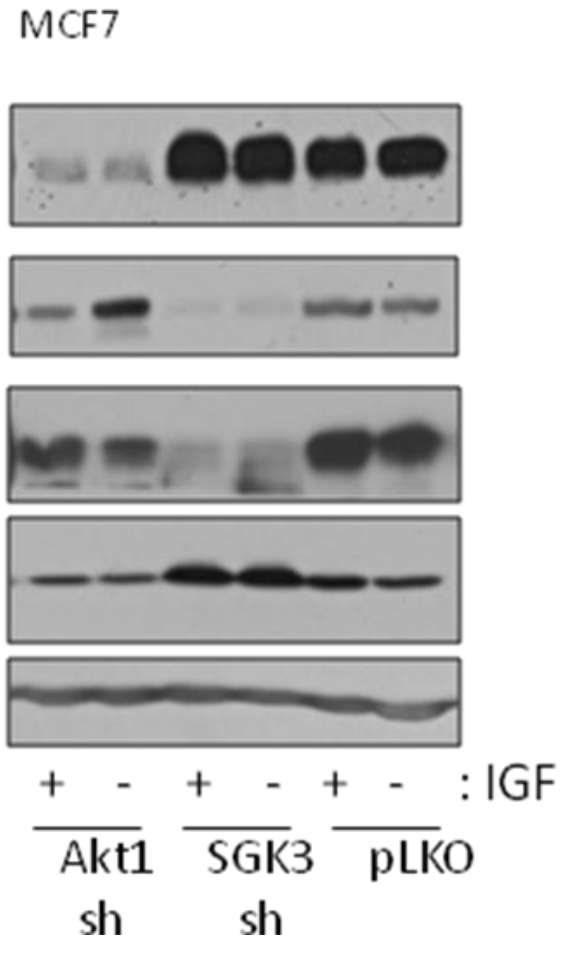

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FIGURE 2

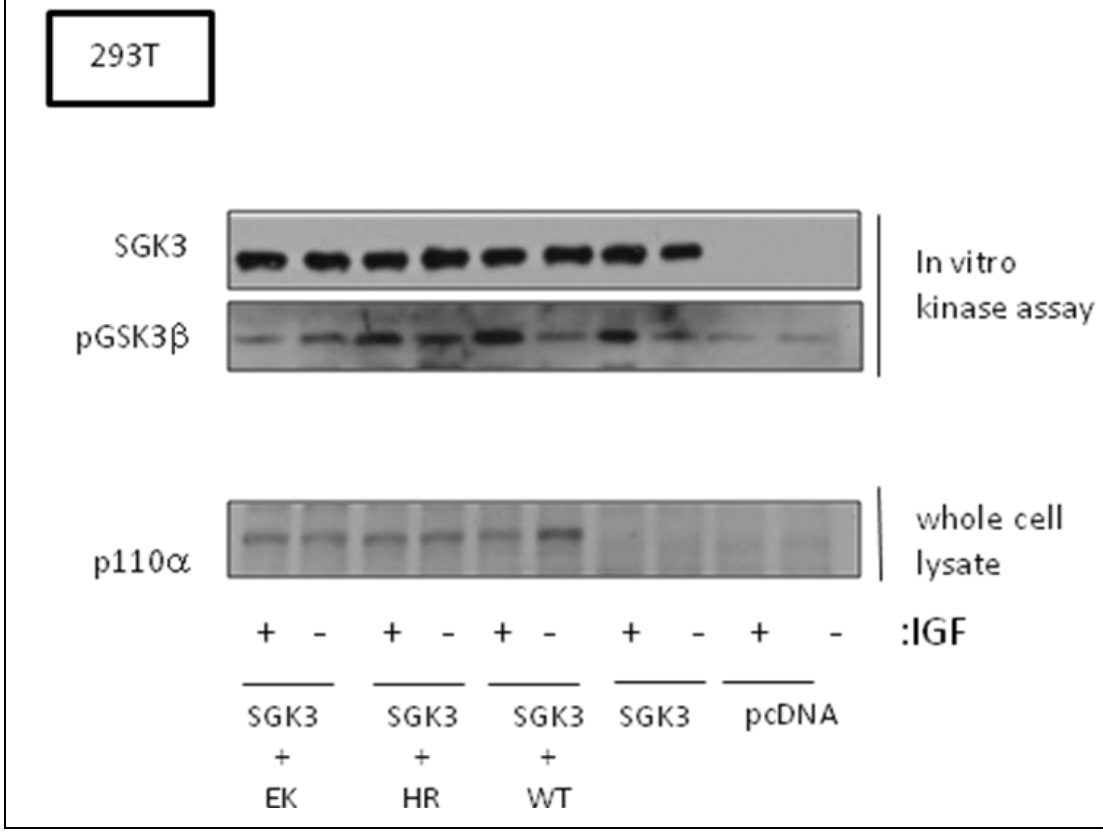


FIGURE 3

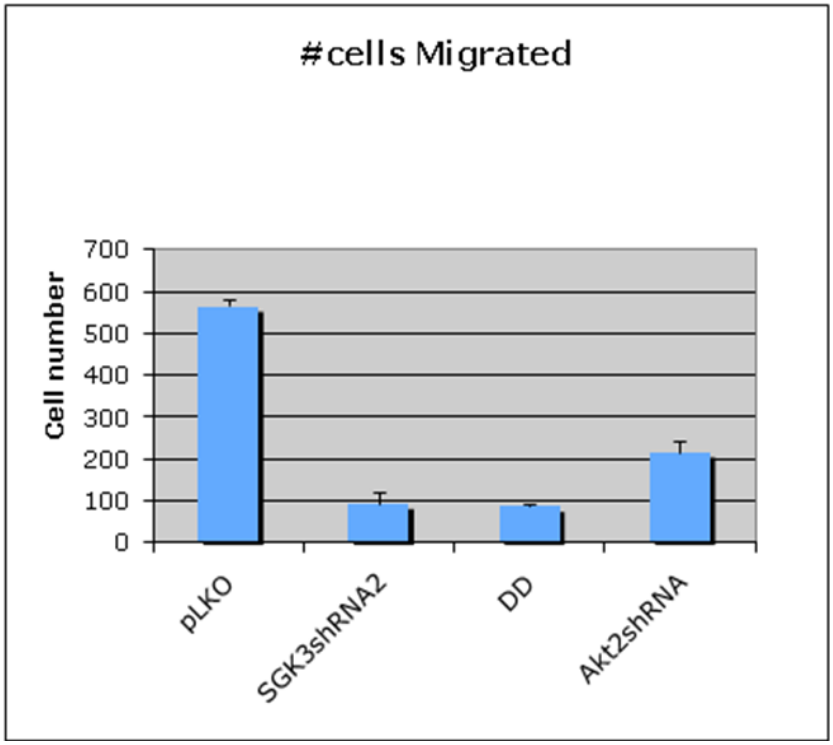


FIGURE 4

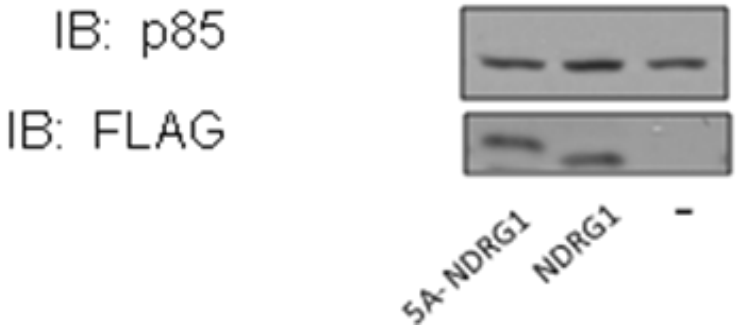


FIGURE 5

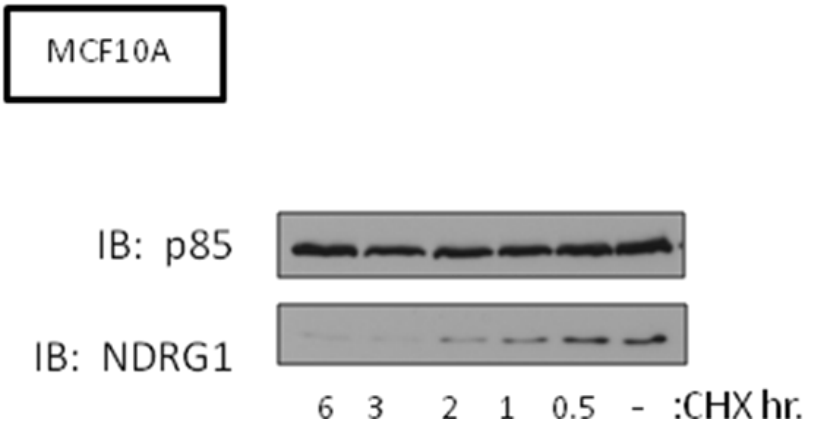


FIGURE 6

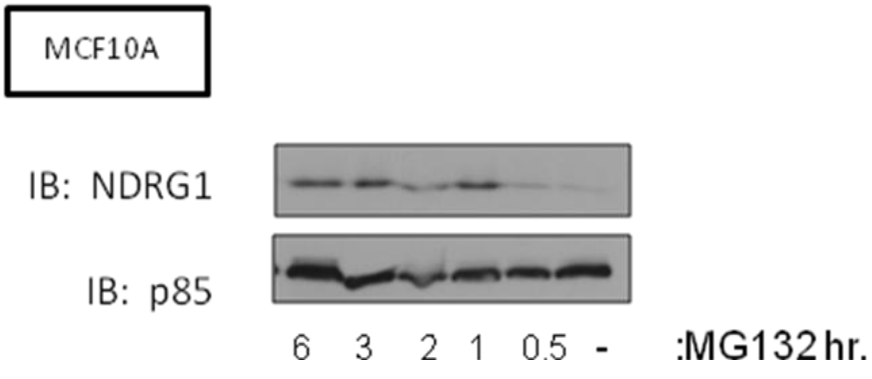
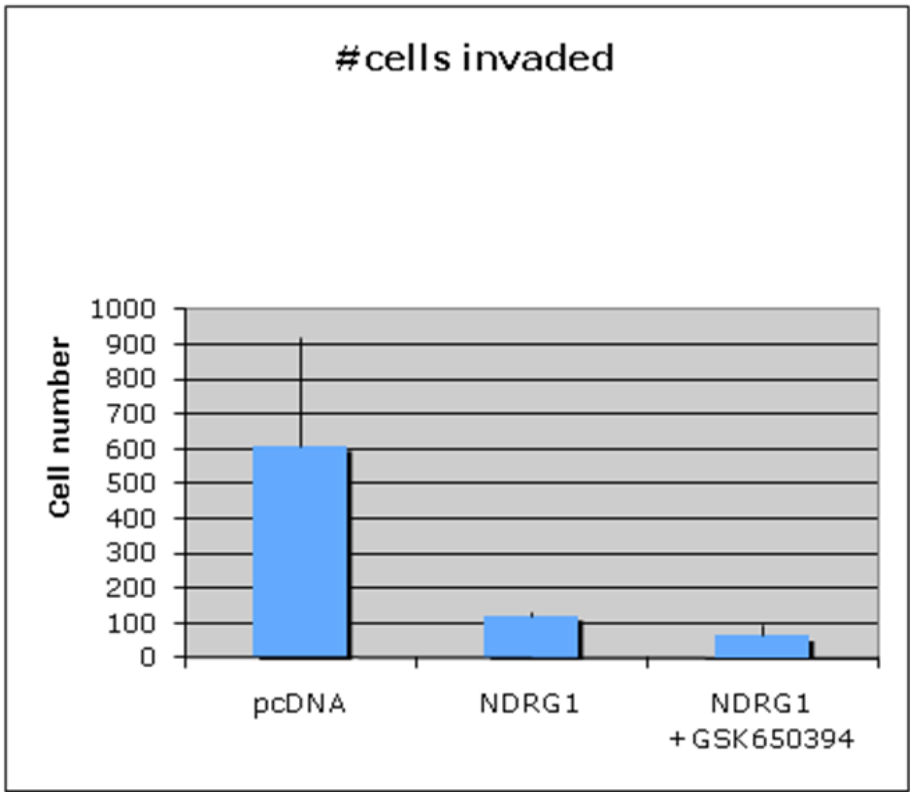


FIGURE 7



Current research and future directions

Current experimental efforts have focused upon examining the concordant expression of Akt and SGKs and evaluating whether there is a dominance between the kinases or if there is a switch to choose which kinases drive the PIK3CA pathway, which is the commonly mutated in breast cancer. Understanding the ability of NDRG1 to mediate SGK3 phenotypic rolls in the invasive migration of breast cancer cells will be another prominent focus prior to commencement of work in murine models.

Key Research Accomplishments

- **SGK3 silencing decreases NDRG1 phosphorylation and increases total NDRG1**
- **Overexpression of PIK3CA breast cancer hot spot mutations, H1047R and E545K, were shown to significantly enhance SGK3 phosphorylation of substrate GSK3b *in vitro.***
- **NDRG1 stability is decreased with cyclohexamide and increases with MG132 treatment correlating with degradation by the 26S proteasome.**
- **NDRG1 SGK phosphorylation sites were mutated NDRG1-FLAG to T328A/T330A/T346A/T356A/T366A**
- **NDRG1 overexpression causes a decrease in MCF7 cell migration which is enhanced with SGK inhibitor GSK650394**

Reportable Outcomes

N/A

Conclusion

SGK1 and SGK3 have been shown in a number of breast cancer cell lines to enhance both migration and invasion using Transwell migrationa and Transwell Matrigel invasion assays. SGK1 and SGK3 shRNAs were subsequently made and validated and will be used to determine the importance of normally metastatic breast cancer cell utilization of SGKs. Additionally PIK3CA overexpression and knockdown constructs were made and validated. The breast cancer hot spot mutations in PIK3CA commonly mutated in breast cancer tumors were shown upon overexpression to activate SGK3 while wild type overexpression caused little or no change in SGK3 activity. The placement of SGK1 and SGK3 downstream of common breast cancer mutations with demonstrated metastatic characteristics make SGK1 and SGK3 promising future molecular targets for breast cancer therapeutics. We further identify SGK3 as a putative mediator of invasive migration through its phosphorylation of NDRG1 leading to its subsequent degradation.

Appendices

N/A

References:

References

1. Karakas, B., Bachman, K. E., and Park, B. H. (2006) *Br J Cancer* **94**, 455-459
2. Bachman, K. E., Argani, P., Samuels, Y., Silliman, N., Ptak, J., Szabo, S., Konishi, H., Karakas, B., Blair, B. G., Lin, C., Peters, B. A., Velculescu, V. E., and Park, B. H. (2004) *Cancer Biol Ther* **3**, 772-775
3. Gustin, J. P., Karakas, B., Weiss, M. B., Abukhdeir, A. M., Lauring, J., Garay, J. P., Cosgrove, D., Tamaki, A., Konishi, H., Konishi, Y., Mohseni, M., Wang, G., Rosen, D. M., Denmeade, S. R., Higgins, M. J., Vitolo, M. I., Bachman, K. E., and Park, B. H. (2009) *Proc Natl Acad Sci U S A* **106**, 2835-2840
4. Yoeli-Lerner, M., Yiu, G. K., Rabinovitz, I., Erhardt, P., Jauliac, S., and Toker, A. (2005) *Mol Cell* **20**, 539-550
5. Tessier, M., and Woodgett, J. R. (2006) *J Cell Biochem* **98**, 1391-1407
6. Sahoo, S., Brickley, D. R., Kocherginsky, M., and Conzen, S. D. (2005) *Eur J Cancer* **41**, 2754-2759
7. Kachhap, S. K., Faith, D., Qian, D. Z., Shabbeer, S., Galloway, N. L., Pili, R., Denmeade, S. R., DeMarzo, A. M., and Carducci, M. A. (2007) *PLoS One* **2**, e844